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Bwassiwè Hele
a) Laboratory of Physiology/ Pharmacology, Faculty of Sciences, Center of Research and Formation on the Medicinal Plants (CERFOPLAM), University of Lomé, Po Box: 1515 Lomé, Togo.
b) Laboratory of Physiology of Effort, National Institute of the Youth of Physical Education and Sport, University of Abomey Calavi, 01 Po Box: 169 Port-Novo, Benin.

Kossi Metowogo
Laboratory of Physiology/ Pharmacology, Faculty of Sciences, Center of Research and Formation on the Medicinal Plants (CERFOPLAM), University of Lomé, Po Box: 1515 Lomé, Togo

Aklesso P Mouzou
Laboratory of Physiology/ Pharmacology, Faculty of Sciences, Center of Research and Formation on the Medicinal Plants (CERFOPLAM), University of Lomé, Po Box: 1515 Lomé, Togo

Kodjo Adi
Laboratory of Physiology/ Pharmacology, Faculty of Sciences, Center of Research and Formation on the Medicinal Plants (CERFOPLAM), University of Lomé, Po Box: 1515 Lomé, Togo

Judith Ahounou
Laboratory of Physiology of Effort, National Institute of the Youth of Physical Education and Sport, University of Abomey Calavi, 01 Po Box: 169 Port-Novo, Benin.

Christian Cognard
Laboratory of Signaling and Transportations Ionics Membranaires (STIM). ERL CNRS/University of Poitiers n° 7368. Pole Biology Health Buil B36/B37, 1 Street Geoges Bonnet, TSA 51106 86073 Poitiers Cedex 9, France.

Kwashie Eklu-Gadegbeku
Laboratory of Physiology/ Pharmacology, Faculty of Sciences, Center of Research and Formation on the Medicinal Plants (CERFOPLAM), University of Lomé, Po Box: 1515 Lomé, Togo

Pierre H Dansou
Laboratory of Physiology of Effort, National Institute of the Youth of Physical Education and Sport, University of Abomey Calavi, 01 Po Box: 169 Port-Novo, Benin.

Kodjo A Aklikokou
Laboratory of Physiology/ Pharmacology, Faculty of Sciences, Center of Research and Formation on the Medicinal Plants (CERFOPLAM), University of Lomé, Po Box: 1515 Lomé, Togo

Correspondence
Bwassiwè Hele
a) Laboratory of Physiology/ Pharmacology, Faculty of Sciences, Center of Research and Formation on the Medicinal Plants (CERFOPLAM), University of Lomé, Po Box: 1515 Lomé, Togo.
b) Laboratory of Physiology of Effort, National Institute of the Youth of Physical Education and Sport, University of Abomey Calavi, 01 Po Box: 169 Port-Novo, Benin.

Effect of the hydro-alcoholic leaves extract of *Paullinia pinnata* (Lin.) on rats skeletal muscle regeneration after contusion injury and phytochemical analysis

Bwassiwè Hele, Kossi Metowogo, Aklesso P Mouzou, Kodjo Adi, Judith Ahounou, Christian Cognard, Kwashie Eklu-Gadegbeku, Pierre H Dansou and Kodjo A Aklikokou

Abstract
The present study aimed to investigate the muscle-protective effect of PPLE against muscle contusion injury. One hundred wistar rats were divided into five groups (n = 20): a control group, an untreated group and 3 treated groups. At day 0, all the animals except control rats were subjected to muscle contusion injury. Control and untreated groups received orally the vehicle while treated groups received the hydro-alcoholic PPLE (100, 200, and 500 mg/kg) once a day, for 14 days. The blood of five animals of each group was collected in order to evaluate the rates of LDH, CK, AST and ALT enzymes. Hydroxyproline content and histopathological analyses were investigated. Phytochemical screening was performed. Only untreated rats showed increased serum concentration and decreased hydroxyproline content. Histopathological analysis showed myotubes formation in the treated groups. Phytochemical analysis revealed the presence of tannins and flavonoids which could have positive effect on the regeneration process.

Keywords: *Paullinia pinnata*, Muscle, Contusion, Regeneration, Rats.

1. Introduction
Mechanical injuries are the most common type of skeletal muscle injuries. Acute skeletal muscle damage determinates fiber disruptions [23, 27], an oxidative stress and some inflammation [13, 18]. The main characteristic of skeletal muscle is its capacity to regenerate after injury, a process which depends on numerous factors including an inflammatory response, a growth factor synthesis and the subsequent activation of endogenous progenitor cells for muscle and a blood vessel reconstruction [16, 34]. The leakage of cells releases specific cytosol enzymes or proteins, such as creatine kinase (CK), myoglobin, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) into the blood was clinically used as muscular damage indexes [39]. It is well known that, in various types of cells such as hepatocytes, cardiac and skeletal myocytes, erythrocytes and brain cells, the AST serum levels is elevated in a wide spectrum of clinical disorders [39]. Skeletal muscle is known to also contain isoenzymes of CK, AST, ALT and lactate dehydrogenase (LDH), which are released into the blood stream following necrosis [20] and their blood concentrations are good indexes of damages.
Collagen or its constituents such as hydroxyproline could be also such an index as the skeletal muscle extracellular matrix (ECM) plays a role in the passive mechanical properties of bundles of fibers and the whole muscle. Its forms a continuous structure throughout the muscle that provides structural support for individual fibers and plays an important role in force transmission between fibers and tendons [38]. The major structural protein of the extracellular matrix is the fibrous collagen, which can change in response to altered use patterns or pathologies. Collagen is the main protein of connective tissues in animals and the most abundant protein in mammals, constituting a quarter of the total protein content and forming a major constituent of skin, bone, tendon, cartilage, blood vessels and teeth [31]. It is present in nearly all organs and tissues and serves to hold the cells together in discrete units [31] and plays an important role in the tissue structure but has a role in many other functions such as cell growth, differentiation, tissue repair, as well as in many pathological malfunctions. Characteristic of the collagen is the presence of hydroxyproline residues needed for the stabilization of its triple helix [25]. With proline, its precursor, they represent 25 to 30 % of the total amino acids forming the collagen. It was found in muscle as 14 % of the dry mass [28] where it is present as networks of fibers.

In the living muscle these fibers resist the over-extensions which may cause damages to the tissue as the force of contraction is transmitted to the tendons.

Among muscle injuries, 90 % consist of bruises or sprains [4]. This interferes with locomotion and performance in sports activities and daily life [21]. The high variability of such injuries has placed hurdles on conducting clinical studies [5] and on determining the most effective treatment [6]. With the aim of minimizing these variables, previous studies have produced muscle injuries invasively, through muscle compression using forceps on animals [10].

Despite these efforts, none of the treatment strategies adapted to date have been shown to be really effective in strictly controlled trials. Most current muscle injury treatments are based on limited experimental and clinical data and/or were only empirically tested therefore no clear conclusions have been reached regarding the best treatment for muscle injuries [12, 17].

Medicinal plants play a vital role in the maintenance of human health throughout the world notably in the Tropics; it is interesting to investigate the properties of *Paullinia pinnata* Lin. (Sapindaceae). In a recent study, the methanol extract of *Paullinia pinnata* root demonstrated a strong stimulatory effect on the proliferation of fibroblasts, skin cells that play a vital role in the closure of wounds [3]. *Paullinia pinnata* Lin. is widely used in Togo for the treatment of skeletal muscle contusion and strain, bone break and inflammatory diseases.[14] Thus, the aim of the present study was to evaluate the experimental effects of a therapeutic application of a PPLE for repairing muscle tissue that had been subjected to a bruising injury.

2. Materials and methods

The administrated doses of the hydro-alcoholic extract of *P. pinnata* leaves in this study were based on previous report by Adinortey [1]. In this study, we haven't positive control because, despite use non steroidal anti-inflammatory drogues, no clear conclusions have been reached regarding the most effective treatment for muscle injuries [6, 12, 19].

2.1. Plant collection, identification and preparation of extract

Fresh leaves of *P. pinnata* weighed 600 g were harvested at Davié-Tsévié in Zio prefecture situated to 35 km of Lomé/Togo. *Paullinia pinnata*, located on latitude 6°23ˈ5" N and longitude 1°12ˈ18" W (using GPS), were collected in April 2014. The plant was authenticated by Dr. KOKOU Komi, Professor, Department of Botany, Faculty of Science (FDS), University of Lomé (UL)/Togo, where voucher specimen reference number Togo 15313 has been deposited in the Herbarium. The leaves were thoroughly washed with water and chopped into pieces under air conditioning drying for a period of one week. The dried leaves were pulverized, weighed and kept in an air tight plastic bag. The powdered plant material (400 g) was extracted with 50 % alcohol and water (2 X 2 L), by cold maceration for 48 hours with the aid of a mechanical shaker. The mixture was filtered by suction and concentrated using a rotary evaporator R-210 (BUCHI, Switzerland) to dryness. A reddish-brown crude material was obtained and was labeled *Paullinia pinnata* leaves extract (PPLE). A yield of 10.05 % representing 40.2 g was obtained.

2.2. Animal care

Healthy young adult wistar albino rats between 150 and 200 g were obtained from the Animal house of the Department of Animal Physiology, Faculty of Sciences, University of Lomé (UL)/Togo. Both male and female sexes were selected for the study. All the rats were housed in groups in metal cages with soft wooden shavings as bedding and maintained under normal laboratory conditions allowing free access to food and water. The protocol of the study was approved by the Local Ethical Committee for Animal Experimentation of the Department of Animal Physiology, Faculty of Sciences, UL/Togo.

2.3. Muscle contusion model and biochemical indicators measurement

One hundred (100) healthy young wistar albino rats of both sexes were used in this study. At day 0, all the animals except control rats were subjected to muscle injury. After the injury, animals were randomly divided into untreated and treated groups of uniform weight.

One group (n = 20) served as the control, without any intervention, one injured rats group was subjected to injury and remained untreated and 3 groups were subjected to injury and treated with PPLE. Before contusion injury, the animals were anaesthetized with ether inhalation. The posterior region of the animal's legs, around the gastrocnemius muscle, was shelved. The middle part of the muscle belly of the right hind limb of each animal was crushed for 30 seconds by using hemostatic gripper [15]. Sixty (60) minutes after the injury, animals in control and untreated groups received orally vehicle 10 mL/kg of body weight (bw) while the 3 treated groups received, by the same route, the hydro-alcoholic PPLE (100, 200, and 500 mg/kg bw) respectively once a day, for 14 days.

At specific time points after injury (1; 3; 7 and 14 days), blood of five animals under anesthesia of each group was collected by using a capillary tube, from the retro orbital sinus in hemolysis tubes. Blood collected was centrifuged immediately at 3000 revolutions/min during 10 minutes using electric centrifuge (SPN-400 Shimadzu Scientific Corporation, Tokyo, Japan) which radius measure is 10 cm. The separated serum was immediately analyzed in order to evaluate the various biochemical indicators of muscle injury by measurement rates of CK, LDH, AST and ALT using a detecting kit obtained from Cypress Diagnostics (Langdorp, Belgium). Assays were performed according to the manufacturer's instructions. The AST/ALT ratio was assessed using the average of the activities of these enzymes at specific time points.

At the end of the blood withdrawal, the animals were sacrificed by cervical dislocation. The whole gastrocnemius muscle was dissected from its insertions, and was destined to estimate the hydroxyproline content and histological analyses (n = 5/group).

2.4. Estimation of hydroxyproline

The hydroxyproline content assay was performed according to the method previously described by Woessner [40] with minor modifications in order to estimate the collagen content of the gastocnemius muscles. The dissected part on the middle of the muscle belly was dried in the oven (Ocean, Germany) at 60° C during 12 hours. One hundred milligram's (100 mg) of the dried tissue was used to estimate hydroxyproline content. The sample was hydrolyzed in 1 mL of 6 N HCl at 130° C for 4 hours in glass tubes sealed. The dry hydrolyzed sample was recuperated in 10 mL of distilled water before it was neutralized using 100µL of NaOH (2.5N), 100 µL of $CuSO_4$ (0.01M) and 100 µL of H_2O_2 (6%). The mixture was

homogenized by using the vortex and the extracted impurities were then removed and discarded. All tubes were incubated at 80° C during 5 min. After cooling to the ambient temperature, 400 µL of H_2SO_4 (3N) was added to the diluted sample, followed by a 200 µL addition of para dimethylaminobenzaldehyde (PDAB) 5% in solution. The range of hydroxyproline was formed and treated in the same conditions. After incubation at 70°C for 16 min, the concentration of hydroxyproline in the samples was determined by spectrophotometry (UV-1600PC spectrophotometer, Shimadzu) at 540 nm and was normalized to the mass of the original tissue sample. Hydroxyproline standards (0.0, 0.5, 1.0, 2.0, 4.0 6.0 µg hydroxyproline) provided a standard curve for spectrophotometry. All experiments were performed in triplicate to ensure the accuracy of the observations. Hydroxyproline content was normalized to the mass of the original tissue sample of individual muscles and expressed as µg of hydroxyproline/mg of dry weight muscle.

Hydroxyproline (µg) amount in each sample test tube was determined using the regression curve from the hydroxyproline standards. In this study, the appropriate relationship is:

Sample Hydroxyproline (µg) = (Sample Absorbance + 0.0032)/0.042 with correlation coefficient $R^2 = 0.9998$.

2.5. Histopathological analysis

Specimen samples were isolated from untreated and treated (500 mg/kg bw) groups of rats on day 1, 3, 7 and 14 after contusion injury for histopathological examination. Muscle specimens were immediately fixed in 10 % (v/v) neutral-buffered formalin, and the fixative solution replaced every 2 days until the tissues hardened. Each specimen was embedded in a paraffin block, and thin sections (3 µm) were prepared and stained with hematoxylin and eosin (H&E) for general morphological observations. Slides were examined qualitatively under a light microscope for fibroblasts proliferation, angiogenesis, epithelization, and granulation tissue formation by employing a light to intense scale (+ to +++) stored in jpeg format [2].

2.6 Phytochemical screening

Phytochemical testing was performed to assess the various phytoconstituents present in *P. pinnata.* Chemical tests were carried out using hydro-alcoholic extract to identify various constitutes using standard methods of Trease and Evans [36].

2.7. Statistical analysis

All data were expressed as mean ± SEM. Statistical analysis was performed by Graph Pad Prism 5.0, and comparisons were made using one-way ANOVA. To make comparisons between the groups, Newman-Keuls post-test was used. Hydroxyproline content was performed by linear regression curve. P values less than 0.05 were considered to be significant.

3. Results

3.1. Serum AST

The Figure 1 shows that AST activity increased in the untreated group and in PPLE groups on day one after muscle contusion injury when compared to the control group where it remained at its starting value. The activity of AST increased significantly ($p < 0.001$) to its maximum level in injured group without treatment on day 3, 7 and 14. The shape of the curves clearly indicated that the PPLE induced significant inhibition of the AST activity ($p < 0.05$) at day 3 and 14. At day 7, the inhibition was maximal ($p < 0.01$) in the group treated with 200 mg/kg bw. This response was inhibited significantly ($p < 0.01$ and $p < 0.001$), in groups treated with 500 mg/kg bw at day 3, 7 and 14 compared to untreated group. There was no significant ($p > 0.05$) change in the profile of this enzyme in the 100 mg/kg bw treated group.

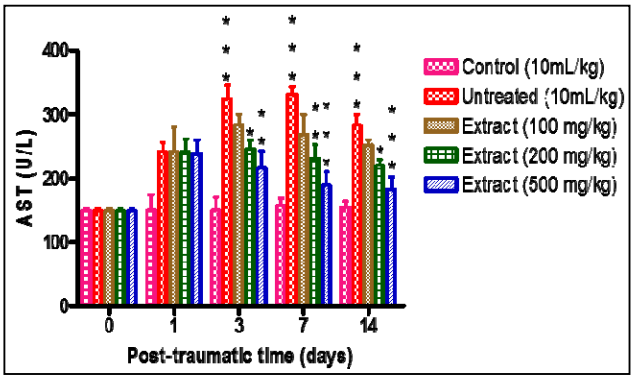

Fig 1: Effects of *P. pinnata* on the AST activity

The values are represented as means ± S.E.M. Statistically significant difference vs control group ***p<0.001. Statistically significant difference vs untreated group: * $p < 0.05$, ** $p < 0.01$,
*** $p < 0.001$.

3.2. Serum ALT

A similar pattern effect was observed for the serum ALT activity. After 24 hours of contusion injury, serum ALT activity increased progressively to it high level at day 3 and 7 in the untreated group compared with the control group which remains at a low level. This increase was inhibited significantly ($p < 0.05$) by treatment with PPLE at dose 500 mg/kg bw on day 3, 7 and 14 compared with the untreated group (Figure 2).

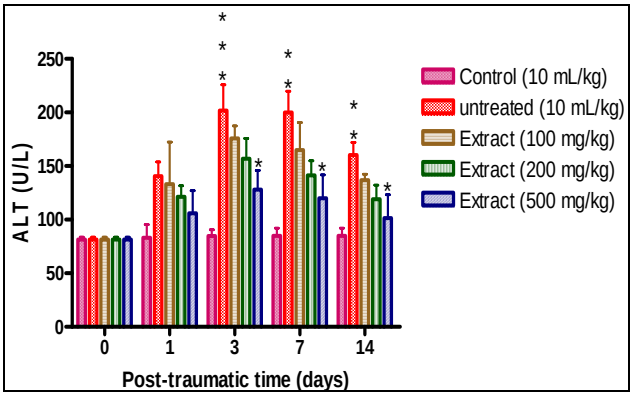

Fig 2: Effects of *P. pinnata* on the ALT activity

The values are represented as means ± S.E.M. Statistically significant difference vs. control group:
** $p < 0.01$, *** $p < 0.001$. Statistically significant difference vs untreated group: * $p < 0.05$.

- **Evolution of the AST/ALT ratio in the time**

The AST/ALT ratio shows an evolution superior to 1 during 28 days treatment in all experimental groups after induction of the muscular bruise (table 1).

Table 1: Evolution of the AST/ALT ratio

Days	Control	Untreated	Doses (mg/kg)		
			100	200	500
1	1.80 ± 2.05	2.14 ± 1.09	1.81 ± 0.99	2.20 ± 2.32	2.24 ± 1.06
3	1.71 ± 1.49	1.91 ± 1.35	1.61 ± 1.45	1.90 ± 3.82	1.91 ± 2.19
7	1.76 ± 1.21	1.94 ± 1.94	1.63 ± 1.24	1.68 ± 0.63	1.75 ± 1.89
14	1.75 ± 0.8	1.74 ± 2.08	1.54 ± 1.53	1.52 ± 5.98	1.60 ± 0.73

3.3. Serum CK

The variation of serum CK activity was also similar to the two precedent enzymes activities, that is, a fast increase on day 1 followed by a slow decrease at later days (day 7 and 14) after injury, with an inhibition effect of PPLE in treated groups with a global acceleration of the inhibition as the level started to decrease as soon as the day 3. On day 1, serum CK activity increased significantly ($p < 0,001$) in the untreated group and PPLE groups compared to the control group. It decreased significantly ($p < 0.05$ and $p < 0.01$) in the PPLE groups 200 and 500 mg/kg of body weight respectively at days 3. At day 7, PPLE (100, 200 and 500 mg/kg of body weight) inhibited significantly $p < 0.05$, $p < 0.01$ and $p < 0,001$ respectively serum CK level. Also, serum CK activity decreased significantly $p < 0.05$ at dose 100 mg/kg and $p < 0,001$ at doses 200 and 500 mg/kg at day 14 compared to the untreated group (Figure 3).

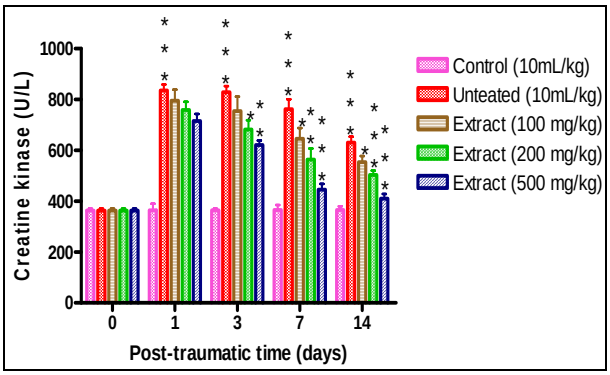

Fig 3: Effects of *P. pinnata* on the CK level

The values are represented as means ± S.E.M. Statistically significant differences vs control group: *** $p < 0,001$. Statistically significant differences vs untreated group: * $p < 0.05$; ** $p < 0.01$;
*** $p < 0.001$.

3.4. Serum LDH

The serum LDH activity level also followed a similar variation shape to the other enzymes, but, this time, at a slower pace. As shown in Figure 4, serum LDH activity increased significantly in the untreated group and PPLE groups ($p < 0.001$) compared to the control group at day 3, 7 and 14 after muscle contusion injury. This increase was significantly inhibited by treatment with PPLE (500 mg/kg of body weight) at day 3 ($p < 0.05$) 7 and 14 ($p < 0.01$). PPLE (200 mg/kg bw), inhibited significantly ($p < 0.05$) LDH level at day 7 and 14, while no difference was noted in the group 100 mg/kg bw, compared to the untreated group.

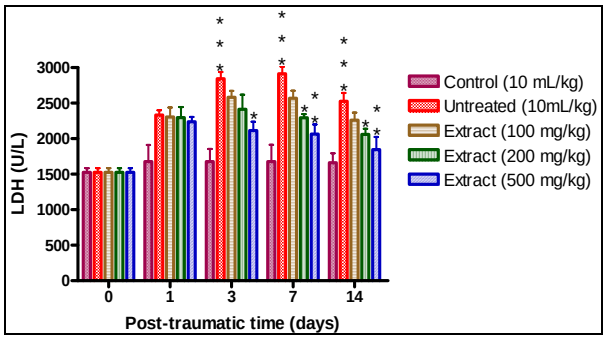

Fig 4: Effects of *P. pinnata* on the LDH release

The values are represented as means ± S.E.M. Statistically significant differences vs control group: *** $p < 0.001$. Statistically significant difference vs untreated group: * $p < 0.05$, ** $p < 0.01$.

3.5. Hydroxyproline content

Hydroxyproline (µg) amount in each sample test tube was determined using the regression curve from the hydroxyproline standards. In this study, the appropriate relationship is:

Sample Hydroxyproline (µg) = (Sample Absorbance + 0.0032)/0.042.

Muscle collagen content variation was determined using a hydroxyproline assay reported in figure 5. Hydroxyproline content displayed a decreased significantly ($p < 0.001$) at day 1 and 3, followed by increase significantly ($p < 0.01$) rebound at day 7 and 14. Hydroxyproline synthesis increase significantly ($p < 0.05$) in PPLE group 500 mg/kg bw compared to the untreated group after muscle contusion injury at day 14.

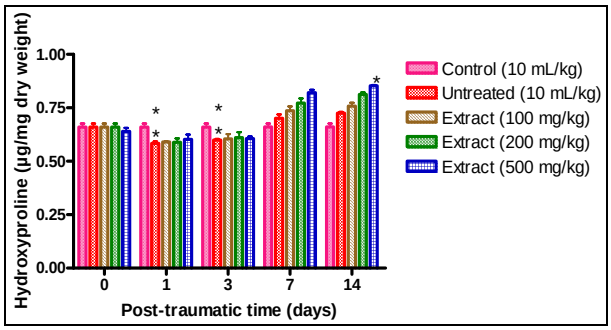

Fig 5: Effects of *P. pinnata* on hydroxyproline content in the muscle injured of rat

The values are represented as means ± S.E.M. Statistically significant difference vs control group:
** $p < 0.01$. Statistically significant difference vs untreated group: * $p < 0.05$.

3.6. Histological analysis

On day 1, 3, 7 and 14 after the injury, the histological sections from the muscles in untreated group showed the presence of cell infiltrate and tissue disorganization. The magnification of x40 made possible to observe myofibers maturation in treated groups and hematoma undergoing a repair process (Figure 6). Greater tissue regeneration was observed in the *P. pinnata* treated groups, as demonstrated by the news fibers proliferation and myotubes formation.

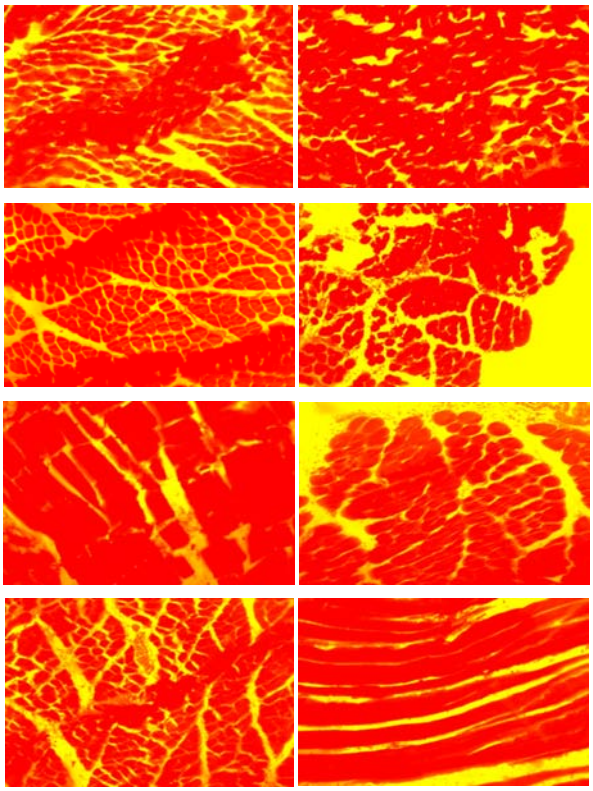

Fig 6: Histological examination of treated and untreated muscle sections stained with H&E. A7 and B14 longitudinal sections, vertical sections: A1, B1, A3, B3, B7 and A14. The photomicrographs show healed muscle sections isolated from rats untreated (A) and treated (500 mg/kg) with PPLE (B) on day 1, 3, 7 and 14 after injury. The photomicrographs were taken at a magnification of × 40. Abbreviation: td: tissue disorganization; ci: cells infiltrate (leucocytes); my: myotube; cn: cells necrosis. nf: news fibers.

3.7. Phytochemical screening

Flavonoids, alkaloids, cardiac glycosides, saponins, tannins, carbohydrates, sterols and triterpenoids tested positive in the leaves extracts of *P. pinnata* (table 2).

Table 2: Phytochemical screening of the *P. pinnata* leaves

Phytochemical Classes	Phytochemical tests	Results
Tannins	Ferric chloride test	+
Flavonoids	NaOH test	+
Saponins	Frothing test	+
Cardiac Glycosides	Molisch test	+
Alkaloids	Mayer's test, Dragendoff's test	+
Steroids and triterpenoids	Lieberman-Burchard's reaction	+
Carbohydrates	Fehling's test	+

+: Presence

4. Discussion

The increase in serum enzyme levels suggests that the contusion protocol used in this study resulted in skeletal muscle injury. The efflux of these enzymes from muscle may occur as a result of increases in the permeability of myocellular membrane [9, 29]. Serum levels of skeletal muscle enzymes or proteins are markers of the functional status of muscle tissue, and vary widely in both pathological and physiological conditions [29].

In the present study we examined the effect of *P. pinnata* on muscle injury *in vivo* which resulted in increases in ALT and AST levels in untreated group. AST and ALT activities were measured to analyze the health status of muscle. Creatine kinase and LDH activity in the serum were also determined as indicators of muscle injury. The high levels of these enzymes observed at the first time decreased progressively during the post-traumatic time in treated groups. These data suggest that, contusion injury may cause pathological damage to the gastrocnemius muscle since, it's well-known that, ALT and AST are present in many tissues like skeletal muscle and it was reported that muscle injury led to transient increases of these enzymes activity, confirmed by Pettersson *et al* [30].

We found that, the AST/ALT ratio was superior to one, during the post-traumatic time in all the groups. This result suggests that the observed increase in transaminase may be due to lesion of myocyte caused by the crushing the muscle. Pratt and Kaplan [32], demonstrated that, when the AST/ALT ratio is superior to one, it is necessary to measure out the CK to confirm the muscular origin of the increase of these enzymes because 90 % creatine kinase (CK) resides in skeletal muscle, located in the cytoplasma. Also, elevated ALT levels in the absence of other evidence of liver disease should lead to consideration of muscle injury, which is confirmed by elevated CK and LDH levels. In this experiment, serum CK was significantly increased after muscle damage. These data indicate that the increase in the measured enzymes in this study resulted from gastrocnemius muscle contusion injury, in accordance with results from previous studies [7, 11, 30].

PPLE administration significantly decreased AST and ALT levels after muscle injury in a dose-dependent way, indicating that *P. pinnata* may protect muscle cells from destruction. In our study, we observed a major elevation of CK level after injury. We found that serum CK was lower in the treated groups than the untreated ones. This could help to reduce the damage caused by the free radical and/or other factors. According to this evidence, many investigators suggest that this enzyme measurement is a basic diagnostic indicator useful in early detection of changes caused by pathological change of the muscle [37].

In the experiments, serum LDH was significantly increased in a similar way to the one of CK after muscle damage, suggesting that a common mechanism may be involved. After administration of PPLE, the serum LDH decreased significantly. This indicates that the extract can improve the status of metabolism and mitigate the damage of membranous structures. Cellular swelling occurred after muscle injury and the microcirculation disturbance caused the accumulation of poisonous matters, which resulted in the increased permeability of cell membrane as well as a serum enzymes increase due to their leakage from muscle cells [37].

It is very interesting that hydroxyproline content decreased in untreated and treated rats the first five days compared to the control group which underwent no specific treatment after the trauma. A possible explanation is that the trauma causes loss of muscle tissue. It also indicates that the injured area of muscle may not gain significant tensile strength until one week after injury in the absence or presence of treatment. Hydroxyproline assay results demonstrated increased collagen content when treated and untreated rats were compared. These results suggest that the accumulated collagen in injuries muscles after contusion aims for restoring the injured tissues.

This confirmed that a certain period, about five days for rat muscle, is required to allow newly-formed granulation tissue to cover the injured area and to have sufficient tensile strength to withstand subsequent mobilization or stretching [26].

Hydroxyproline is an uncommon amino acid present in the collagen fibers of granulation tissues. Measurement of the hydroxyproline could therefore be used as an index for collagen turnover. Biochemical analysis revealed increased hydroxyproline content, which is a reflection of an increased cellular proliferation and therefore an increased collagen synthesis, after *P. pinnata* administration. The sequence of appearance of hydroxyproline observed in this study is similar to that reported to occur in wound healing [3, 22] and in experimental granulation tissue [24]. This work is similar to results in other studies demonstrating that hydroxyproline assay of muscle biopsies from patients with spasticity displayed increased collagen content compared to normal muscle [8, 33]. The reduction of enzymatic markers and enhancement of hydroxyproline after PPLE administration treatment were correlated with the histopathological findings of an increased fibroblasts proliferation, new fibers and myotubes formation.

The phytochemical constituents are physiologically active compounds possessing great potential for therapeutic and prophylactic uses. Recent studies revealed that phytochemical constituents such as flavonoids, triterpenoids, saponins and tannins can promote the muscle healing process which compounds was found in *P. pinnata* leaves extract. This result confirmed previous study revealed by Havsteen [19] and Souza *et al* [35].

The potent muscle regeneration capacity of PPLE, as evidenced by an increased hydroxyproline synthesis and decreased levels of enzymatic parameters in regenerating tissue, validates the ethnotherapeutic claim.

5. Conclusion

The results revealed the potential use of PPLE as an internal treatment for muscle reconstruction. The mechanism of action of PPLE was postulated to involve biochemical markers (AST, ALT, CK, LDH) reduction, angiogenesis, collagen deposition, tissue formation and myofibers maturation at the proliferative stage. These actions are attributed to the synergistic effects of the active compounds in the extract such as flavonoids, saponins, tannins and phenols revealed in this study. Overall, *P. pinnata* appears to promote muscle regeneration and to accelerate motor functional recovery by reducing enzymes activity and increasing hydroxyproline synthesis. However, further study is needed to isolate the active components that promote muscle regeneration before PPLE can be used clinically. It is recommended that further studies into specific phytochemical constituents of plant extract regulate integrity of muscle tissue be carried out.

6. References

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